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Cyclosporine inhibition of a murine B cell lymphoma

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SUMMARY

The effect of cyclosporine (CsA) on the CH12 murine B cell lymphoma was investigated to determine whether sensitivity to this agent is retained by malignant B cells. This tumour produces an antibody to bromelain-treated red blood cells and may represent transformation of a B cell with certain activation properties associated with early resting B cells. In *in vitro* cultures, the growth and proliferation of CH12 were inhibited by CsA in concentrations of 0·1–1·0 μg/ml; these levels were ineffective against non-lymphoid tumours, although some non-specific cell toxicity was noted at higher levels. IgM antibody production, as measured by enzyme-linked immunosorbent assay (ELISA), was inhibited over the same range. CH12 cells stimulated by lipopolysaccharide, however, were less sensitive to CsA than untreated cells. These studies indicate that malignant B cells may be sensitive to CsA, perhaps reflecting their derivation from a functionally distinct B cell population with enhanced drug sensitivity.

Keywords cyclosporine B cell lymphoma immunosuppression

INTRODUCTION

Cyclosporine (CsA) is a fungal metabolite with novel immunosuppressive properties. This agent acts selectively on lymphoid cells and suppresses immune function primarily by clocking the production and response to interleukin 2 by T lymphocytes (Borel *et al.*, 1977; Britton & Palacios, 1982; Calne, 1979; Cohen *et al.*, 1984). The inhibition of cellular immune function without myelotoxicity has contributed to the value of CsA in the treatment of organ graft rejection. CsA displays other actions that may be useful in immunological studies as well as the clinical setting. In both human and murine systems, CsA can directly inhibit B cell proliferation and antibody production independent of T cell influence (Paavanen *et al.*, 1981; Delespesse *et al.*, 1983; Berger, Meingassner & Knapp, 1983; Muraguchi *et al.*, 1983). The interference with B cell function occurs at an early stage in activation, although in the mouse, interestingly, it is not uniform among B cell populations. Thus, CsA preferentially affects murine B cells responding to stimulation with anti-μ as well as certain T cell independent antigens (Kunkl & Klaus, 1980; Dongworth & Klaus, 1982). The *in vitro* production of anti-DNA autoantibody by murine spleen cells has also been shown to be more sensitive to CsA than other antibody responses (Mayus, Semper & Pisetsky, 1985). These observations have suggested that CsA can selectively modify certain antibody responses, serving as pharmacologic marker of functional B cell populations.

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The suppressive action of CsA for B and T lymphocytes has also led to interest in its use as a selective agent in lymphoproliferative disorders. Indeed, CsA was shown to inhibit the *in vitro* proliferation of long-term human T cell tumour lines. This effect was demonstrated at dose levels which were inactive against other non-T cell hematopoietic cell lines, including human and monkey B cell lines as well as a murine plasmacytoma (Fou *et al.*, 1981; Berger *et al.*, 1982). In other investigations, CsA failed to inhibit the growth of Ebstein-Barr virus transformed human B cells (Graham Bird *et al.*, 1981). These results have suggested a selective anti-proliferative activity against T cell tumours among transformed lymphoid cells, although the number of B cell lines examined thus far has been small.

We have therefore questioned whether greater inhibitory activity against B cell tumours could be demonstrated among other lines, particularly those derived from populations which normally have enhanced CsA sensitivity. To investigate this possibility, we analysed the response to CsA of a B cell tumour termed CH12 (Lanier *et al.*, 1982; Arnold *et al.*, 1983; LoCascio *et al.*, 1984). CH12 was derived from a mouse of the B10.A-2^{a}H-4^{b}p/wts strain. It bears cell surface IgM and can be stimulated to production of a monoclonal antibody product which binds bromelain-treated mouse red blood cells (RBC) as well as sheep RBC. The presence of the cell surface marker Lyt-1 as well as the production of an autoantibody product (anti-bromelain-treated mouse RBC) suggests that CH12 may be derived from the B cell population with greater CsA sensitivity. Moreover, since CH12 responds to lipopolysaccharide (LPS) as well as T cell signals plus antigen, this tumour demonstrates certain activation properties in common with early resting B cells and may therefore be more drug sensitive. In this communication, we present evidence that CsA blocks spontaneous antibody production and proliferation of CH12 cells *in vitro*. These results indicate that sensitivity to CsA may be retained by malignantly transformed B cells and suggests the use of CH12 as a model to investigate the anti-proliferative activity of CsA against B cell tumours.

MATERIALS AND METHODS

Cells and mice. CH12 cells were maintained in ascites form in strain B10.A mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). In some experiments, cells were briefly passed in culture prior to use. The following cells lines used as controls were provided by Dr J. Brice Weinberg, Durham VA Hospital: U937 (human monoblastic cell), K562 (human erythroid/chronic myeloid cell) and HeLa (human epithelial carcinoma).

Cyclosporine and mitogens. The powder form of CsA was the generous gift of Sandoz Pharmaceuticals (East Hanover, NJ, USA). A stock solution of 2 mg/ml was prepared by first dissolving the powder in 0·25 ml of 96% ethanol. Following addition of 0·1 ml of Tween 80, the volume was brought to 3·5 ml with phosphate buffered saline (PBS). LPS from *E. coli* 0127.B8 (Difco Laboratories, Detroit, MI, USA) was used at a concentration of 2·5 μg/ml while Concanavalin A (Pharmacia, Uppsala, Sweden) was used to a concentration of 5 μg/ml.

Cell cultures. CH12 cells, obtained by ascites tap or from passage in culture, were first washed with Dulbecco's modified Eagle medium (DMEM) media and then suspended in RPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 2×10^{-5} M 2-mercaptoethanol and antibiotics. For these experiments, T cell depleted populations were obtained by treating cells with two monoclonal anti-T cell reagents HO-13-4 recognizing Thy-1.2 (Marshak-Rothstein *et al.*, 1979) and C3PO recognizing Lyt-1.2 (Mark *et al.*, 1982) followed by guinea pig serum as complement source. Although CH12 displays the Lyt-1.2 antigen, it was possible nevertheless to use C3PO for T cell killing since CH12 viability was unaffected by this antibody at the concentrations used. After counting, cells were diluted usually to 5×10^{4}–5×10^{5} cells/ml in media with or without CsA or LPS. For antibody assays, cells were cultured in 24 well plates (Linbro, Flow Laboratories, McLean, VA, USA) using a total volume of 0·6 ml. For proliferation assays cells were cultured using 96 well microtitre plates with a final volume of 0·2 ml. Cells were incubated for 5–7 days at 37°C in 7% CO_2 atmosphere before harvesting. Viable cell counts were determined by microscopy after staining with Trypan blue.

Antibody assays. Total IgM levels were determined by enzyme-linked immunosorbent assay

(ELISA) as described elsewhere (Mayus *et al.*, 1985). Briefly, Dynatech microtitre plates were coated with the $F(ab')_2$ fragment of goat anti-mouse IgG ($F(ab')_2$ specific; Cappel Laboratories, Cochranville, PA, USA) at 3 μg/ml in PBS. Plates were then washed with PBS containing 0·05% Tween 20 (PBS-T) and then incubated with various dilutions of culture supernatant for 1 h at room temperature. Following additional washing, plates were exposed sequentially to a 1:4000 dilution of rabbit anti-mouse IgM (Litton Bionetics, Charleston, SC, USA) for 1 hr at room temperature and then peroxidase conjugated goat anti-rabbit IgG (Litton Bionetrics). The final step after washing with PBS-T was incubation with the peroxidase substrate 3,3′,5,5′,-tetramethylbenzidine in pH 4 citrate buffer containing H_2O_2. Optical densities were determined at 380 nm using a Titertek Multiskan platereader. Results are reported in terms of OD380 units.

Proliferation assay. After culture, cells in microtitre plates were pulsed for 6 h with one μCi methyl-^{3}H-Thymidine (2Ci/mm, Amersham, Arlington Heights, IL, USA) per well. The cells were harvested onto glass paper using a 24 channel microharvester (Bellco Glass, Vineland, NJ, USA) and radioactivity measured in Aquasol-2 counting fluid (New England Nuclear, Boston, MA, USA) using a Packard 3375 liquid scintillation spectrometer. The mean and standard deviation of quadruplicate cultures were obtained.

RESULTS

The activity of CsA on CH12 was tested *in vitro* measuring proliferation by tritiated thymidine incorporation as well as antibody production by ELISA. Figure 1 shows results of a representative experiment demonstrating that CsA caused a profound decrease in thymidine incorporation achieving 50% inhibition at approximately 100 ng/ml with complete inhibition at 1·0 μg/ml. These dose levels are comparable to those reported to inhibit T cell lines (Berger *et al.*, 1982). The action of CsA on CH12 was also reflected in viable cell counts which decreased over the duration of this experiment. Furthermore, CsA inhibited the spontaneous production of antibody by CH12 over the same dose range (Fig. 1). Since this tumour was maintained by passage in animals, we questioned whether some of the CsA inhibition resulted from inhibition of growth factor production by T cells present among the tumour. The preparations obtained from the animals failed to show stimulation by concanavalin A. Moreover, the experiments were repeated with tumour cell preparations depleted of T cells by treatment with monoclonal anti-T cell reagents which were inactive against CH12 despite its expression of Lyt-1. These preparations showed the same sensitivity to CsA as untreated populations (data not shown), arguing against an indirect effect of CsA.

It was important next to establish that the antiproliferative effect of CsA on CH12 did not result from non-specific toxicity to cultured cells. Although previous experiments demonstrated resistance

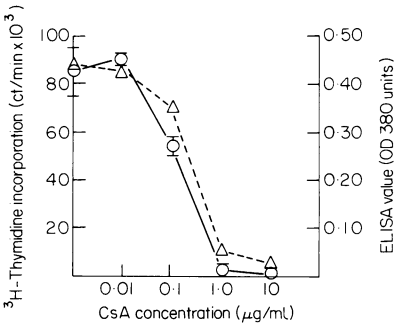

Fig. 1. CsA Inhibition of CH12 proliferation and antibody production. CH12 cells were grown in tissue culture with various concentrations of CsA. Proliferation was measured by the incorporation of tritiated thymidine (○) while antibody production was measured by ELISA (△). CH12 cells were present at 5×10^5 cells/ml.

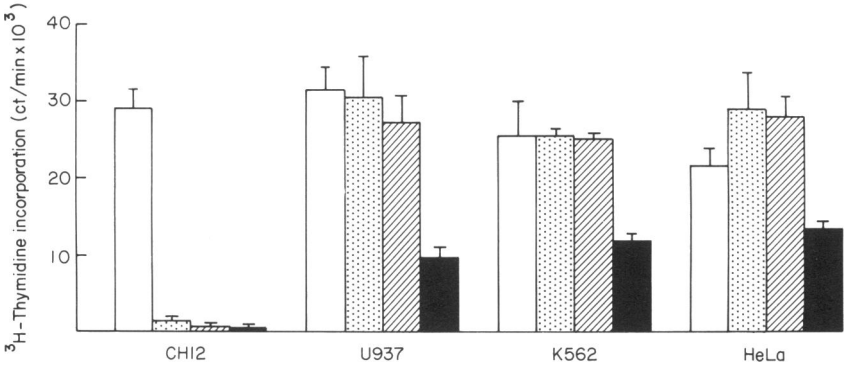

Fig. 2. CsA inhibition of tumour cell lines. The effect of CsA on proliferation of tumour cell lines was tested using CH12, U937, K562 and HeLa cells. (□) Proliferation in the absence of CsA; (▨, ■, ■) proliferation in the presence of 0·1, 0·5 and 5 μg/ml of CsA respectively. Cell concentrations were selected to give equivalent degrees of tritiated thymidine incorporation and varied for the different cell lines, including CH12 (10^5 cells/ml) U937 (5×10^4 cells/ml), K562 (10^4 cells/ml) and HeLa (10^4 cells/ml). Inhibition patterns were independent of cell numbers, however, over a five-fold range.

of many cell lines to CsA (Berger *et al.*, 1982) we retested a series of cell lines under our culture conditions. As shown in Fig. 2, at doses of CsA which completely blocked proliferation of CH12, three other cell lines were unaffected. Some inhibition of proliferation was noted, however, at very high doses of CsA although this inhibition was only partial and required concentrations of CsA 10- to 50-fold greater to achieve levels of inhibition comparable to that observed for CH12.

In normal B cells, stimulation by LPS produces resistance to CsA presumably by providing a signal for activation that bypasses a CsA sensitive step (Klaus & Hawrylowicz, 1984). Since CH12 responds to LPS, we tested the influence of this mitogen on CsA sensitivity. As shown in Fig. 3, cells stimulated by LPS were more resistant to CsA than unstimulated cells, an effect observed with antibody production as well as proliferation. This effect was observed, moreover, over a range of cell numbers tested (5×10^4 to 5×10^5). Thus, as in the case of normal B cells, CH12 sensitivity may reflect the nature of the signal producing activation.

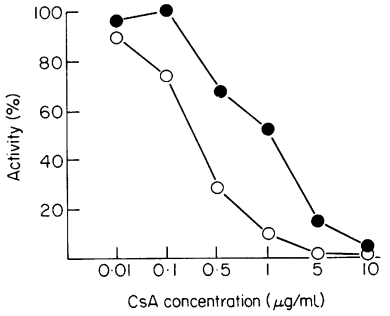

Fig. 3. Effect of LPS on CsA sensitivity of CH12. The sensitivity of CH12 proliferation to CsA was tested with and without 2·5 μg/ml of LPS. In these experiments, LPS was present from the initiation of culture, with proliferation determined after 5 days. (○) Activity without LPS; (●) activity with LPS. Each point represents the average of determinations from two separate experiments. Proliferation values for cultures without LPS were 46,253 ± 2350 and 69,190 ± 4083 while those with LPS were 89,175 ± 4709 and 124,929 ± 4162.

DISCUSSION

These studies provide further evidence for a direct action of CsA on B cells and indicate moreover that sensitivity to this agent may be retained by malignantly transformed cells. These findings parallel studies on T cell lines whose *in vitro* proliferation were inhibited by CsA (Fou *et al.*, 1981; Berger *et al.*, 1982). Together, they suggest that monoclonal tumour cell preparations of B as well as T cell origin may be useful for delineating the mechanisms of CsA action. In addition, they indicate that CsA may be an effective probe for growth regulation in selected lymphoid malignancies.

At least two mechanisms can explain the inhibition of CH12 growth and function by CsA. The first is that CH12 is derived from a B cell population with intrinsic sensitivity to this agent. There is a difference among B cell populations in their response to CsA with those responding to certain T-independent antigens preferentially inhibited (Kunkl & Klaus, 1980; Dongworth & Klaus, 1982). There is evidence moreover that autoantibody producing B cells are more sensitive to CsA than others (Mayus *et al.*, 1985). CH12 produces an antibody product to bromelain-treated mouse RBC that is sometimes characterized as an autoantibody. In addition, CH12 bears the cell surface marker Lyt-1 which is expressed by autoantibody producing B cells (Hayakawa *et al.*, 1984). The simplest interpretation of these findings is that growth inhibition of CH12 by CsA reflects its origin from a B cell population which characteristically enhanced sensitivity to this agent.

An alternative explanation is that CH12 is in a state of activation or differentiation that is CsA sensitive although not dependent on its derivation in a particular B cell population. CH12 has features suggestive of an early resting B cell and retains responsiveness to certain signals for stimulation. This functional state may be the determinant of CsA sensitivity. It is noteworthy that treatment of CH12 with LPS reduces sensitivity to CsA. This result suggests that stimulation by mitogen bypasses an activation step that is the target site of CsA. In this view, the sensitivity of CsA may be dependent on the activation pathway of a cell rather than membership in a particular population. It will be of interest therefore to determine the scope of B cell tumours that are inhibited by CsA in both human and murine systems and the cell functional markers that are associated with this property.

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